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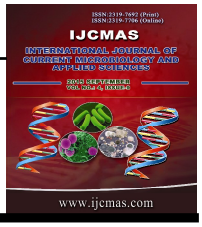

Original Research Article

Isolation and Characterization of Nickel and Cadmium Accumulating Bacterial Isolates from *Mystus cavasius and Glossogobius giuris* of River Hooghly

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K e y w o r d s

Mystus cavasius, *Glossogobius giuris*, Nickel, Cadmium, Bio-accumulation, River Hooghly

A B S T R A C T

Heavy metal pollution for the last few decades is spreading throughout the world along with rapid industrialization. A large number of bacteria and their products can be highly efficient bio-accumulators of different forms of metals. The present study deals with isolation and characterization of nickel and cadmium tolerant and accumulating bacteria isolated from the guts of *Mystus cavasius* and *Glossogobius giuris* of river Hooghly. The two potential metal tolerating isolates were morphologically, physiologically and biochemically characterized. The study indicated the potentiality of the isolates SAT-1 and SAT-2 to tolerate and accumulate significance amount of both nickel and cadmium. The amount of nickel and Cadmium accumulation from fish flesh samples of *Mystus cavasius and Glossogobius giuris* were also analyzed. The present study indicates an alarming amount of heavy metal content in tissue of both fish.

Introduction

Presence of heavy metals in the industrial waste water is a potential hazard to aquatic system, animal and human. High concentrations of heavy metals often pose a serious threat to biota and the environment of any ecosystem (Cheng, 2003). Heavy metal pollution can be a much more serious problem because they cannot be degraded by natural processes and persist in soil and sediment from where they are released gradually into water bodies as sink, In River Hooghly nickel contamination and nickel accumulating bacterial isolates were found (T. Mukherjee et al.2014). Distributions of such heavy metals through food chain affect ecological community. Microorganisms, owing to their large surface volume ratio and high metabolic activity, are important vectors in introducing heavy metals into food chain. In order to fully assess the risks from fish and fish products, it is important to have information on the incidence of these pathogens (Davies *et al.*, 2001). The occurrence of antibiotic-resistant pathogenic bacteria in surface waters and aquaculture environments is also a well-known phenomenon that carries a negative impact for public health and for the safety of the fish supply (González *et al.* 1999; Toroglu *et al.*, 2005). Recent studies showed that

seafood and fish products are quite often contaminated (Ayas *et al.,* 2007; Samanta *et al.,* 2005). The presence of antibiotic-resistant bacteria in fish throughout the world has been documented in several publications (Nonaka and Suzuki, 2002; Matyar *et al.,* 2004). Heavy metals entering the fish have a possibility to get accumulated in different parts of the body and the residual amount can build up to a toxic level (Kumar and Achyuthan, 2007; Ayas *et al.,* 2007; Yoon *et al.,* 2008). The presence of microorganisms in internal fish organs could indicate the breakdown of immunological defense mechanisms. Although there is no certainty about the existence of strictly aquatic bacteria, the most widespread opinion among the various authors is that the majority of the bacteria found in aquatic environments is of soil origin and carried into the water due to rain or to accidental introduction of natural or a direct consequence of human activity. However, every water mass has its bacterial community, although these communities may vary greatly in both the present groups and the number of cells (Sousa *et al.,*1996). Microorganisms are widely distributed in nature and are found mostly in natural water. In urban and densely populated rural areas, the microbiological quality of fresh water is frequently threatened by contamination with untreated domestic wastewater (Griesel *et al.,* 2002).

Antibiotic resistant microorganisms may be associated with reduced penetration of the antibiotic into the cell, or can results from active processes such as changes in the transport of those compounds into or from bacterial cells (Hermansson *et al.,* 1987). The present study helped us in obtaining more meaningful and realistic knowledge of isolation and characterization of both nickel and cadmium accumulating microbes from river Hooghly and accumulation of cadmium and nickel in *Mystus cavasius* and *Glossogobius giuris* obtained from river Hooghly.

Sample collection

10 fish samples of each of the two species were collected from Belur region of Hooghly River (Fig. 1) and packed in sterile plastic bags on ice. Samples were immediately transported to the laboratory and processed within 30 minutes of their collection. Fishes were externally washed with sterilized water to reduce potential contamination with skin bacteria. Intestines were taken from each fish aseptically, weighed and homogenized in a sterile glass homogenizer and then transferred to a sterile vial containing 100 ml of sterile 0.85% NaCl prepared in deoinized water and agitated at 110 rpm for 30 minutes.

Physico-chemical analysis of water samples

The river water temperature, pH were recorded on the spot. Electrical conductivity, biological oxygen demand (BOD), dissolved oxygen (DO), Total and fecal coliform count were analyzed according to standard methods (APHA, 2005). The nickel and cadmium load of the experimental site fresh river water was measured at SGS enterprise (Kolkata) using standard ICOPES protocol

Isolation of Nickel and Cadmium tolerant bacteria

After allowing the homogenized suspension to stand for 5 minutes, the bacterial isolates were isolated by dilution plating of the supernatant (0.2 ml) in Nutrient Agar (NA) media plates (sterile, 90 mm diameter) (gms / ltr.: Peptic digest of animal tissue 10, Meat extract 10, Sodium chloride 5, agar 15; final pH 7.2±0.2 at 25°C) containing varied concentrations of Ni^{+2} and Cd^{2+} (mM: 0.5,

1,2,3,4,5,6,7,8,9,10) as analytical grade salts of $NiCl_2$ and $CdCl_2$ from their sterilized stocks (100mM). Plates were incubated at 35±2°C for 24 hours. Nickel and Cadmium tolerant bacterial colonies that developed at highest concentration of Ni^{+2} and Cd^{2+}supplemented media were selected as Ni and Cd tolerant isolates for further experimental study. The isolates were maintained as axenic cultures by several periodic subculturing onto Ni^{+2} and Cd^{2+}supplemented Nutrient Agar slants.

Heavy metal tolerance assay

Minimum inhibitory concentration (MIC) of the selected isolates, for the test metals viz. Ni^{+2} and Cd^{2+}were determined using standard tube dilution techniques using Luria broth (gm/ltr.: Tryptone 10, yeast extract 5, NaCl 10; pH 7.2±0.2 at 25°C) supplemented with different concentrations of Ni^{+2} and Cd^{2+}respectively. Overnight (18-20 hours) bacterial inoculations (parameters as stated before) were used as inoculant. Growth were recorded after 24 hours of incubation at 35±2°C (at 110 rpm for broth cultures). The lowest concentration of metal that completely inhibited microbial growth was considered as the MIC. Development of growth indicated by turbidity in the broth medium was observed and accordingly the MIC was determined.

Assay for Determination of accumulated heavy metal in the cells of bacterial isolates

0.25 ml of overnight grown (18-20 hours) cell suspensions were inoculated to 75 ml of TMMG (Tris Minimal medium supplemented with glucose [TMMG], (gm/ltr.:Tris base: 6.05, glycerol -2-phosphate: 0.67, $(NH_4)_2SO_4$: 0.96, KCl: 0.62, $MgSO_4$: 0.063, $FeSO_4$: 0.0003, glycerol: 0.6; 0.8% glucose; pH ~7±0.2 with 2 M HCl) medium in 250 ml conical flasks having 2 mM of Ni or Cd incubated on a shaking incubator (110 rpm) at 35±2°C. Cells were harvested at 24, 48, 72 and 96 h of incubation by centrifugation (6000 g, 10 min, and 4 °C). To analyze the metal content, the medium supernatants were acid digested with nitric acid and perchloric acid (5:3) and the volume was adjusted to a known amount. Nickel and cadmium content in the supernatants was determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). Sets where cells grew without Ni^{+2} and Cd^{2+}were considered as control. The same protocol was applied to the cell biomass to corroborate the results.

Antibiotic sensitivity test

To determine sensitivity to antibiotics, the bacterial strain was tested for its resistance to 9 different antibiotics. The sensitivity to antibiotics was determined by disc diffusion method (Bauer *et al.*, 1966). The bacterial strain was grown on Mueller-Hinton broth (MH, Himedia) at 37 °C for 24 h. Young culture was plated on MH agar plates to have uniform bacterial lawn. The antibiotic discs were placed on freshly prepared lawn of the isolate on MH agar plates and incubated at 37 °C for 48 h. The diameter of the inhibition zones were measured and the bacterium was classified as sensitive (S), resistant (R) and intermediate (I) depending upon the size of inhibition zone following the standard disc sensitivity method (HiMedia, India). The antibiotics used are Tetracycline (30 µg), Imipenem (10 µg), Polymyxin–B (30 units), Ticarcillin (75 µg), Gentamycin (10 µg), Amikacin (30 µg), Nitillin (30 µg), Colistin (10 µg) and Ciprfloxacin (5 µg).

Heavy metal assessment of fish flesh

samples

Samples were thoroughly washed with Mili-Q water after removing the scales, and muscle portion, which was taken for further processing. Muscle tissue was oven dried at 110 ^{0}C, powdered with pestle and mortar and was stored until chemical analysis. Heavy metals (Ni and Cd) were analyzed after digesting the homogenized samples in a mixture of concentrated nitric and perchloric acid [13]. Digestion was carried out after 0.5 gm homogenized powdered sample was placed in a Teflon beaker and digested with a few drops of sodium chloride solution (30%) and a 10 ml mixture (1:5) of concentrate Nitric acid (65%) and concentrated perchloric acid (70%). The free chlorine developed loosens the chemical bonds in organic compounds after gentle heating (at 70±5°C) in a water bath for 12 hrs and destroy the organic matter in order to transfer the metals into the solution. The digested samples were centrifuged and the supernatant was analyzed were using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA)

Results and Discussions

Screening and biotyping Nickel and Cadmium resistant isolates

Heavy metal resistant bacterial colonies were found in all the samples ranging up to 10 mM concentration of Ni^{+2} and up to 7 mM of Cd^{2+} in solid slant assay. Initially 23 different colony isolates had been identified of which 2 isolates (showing a significant high level of heavy metal resistance) have been selected from *Mystus cavasius (SAT-1)* and *Glossogobius giuris (SAT-2)* respectively for the current study. Isolates were characterized biochemically as given in table 2.

Heavy metal tolerance assay

MIC of both the isolates in LB medium were determined for Ni^{2+} and Cd^{2+}. Selected isolates shows high level of tolerance of which (SAT-2) have MIC value of 2mM for Ni^{2+} and 6mM for Cd^{2+} in LB while (SAT-1) shows a significant 9 mM for Ni^{2+} and 7mM for Cd^{2+} value of MIC in LB as shown in table 3 and table 4 indicates the growth of isolates SAT-2 and SAT-1 at their MIC

Nickel and Cadmium removal assay

Figure 2 and 4 indicates that with time the nickel and cadmium content in the bacterial pellet increased significantly. The result shows that both the isolates were nickel as well as cadmium accumulators and the study revealed that isolates (SAT-1) and (SAT-2) accumulates 57.45% and 31.60% of nickel and 54.84% and 59.82% of cadmium respectively after a period of 96 hours. Figure 3 and 5 indicates, the percentage of nickel and cadmium being removed from the medium supernatant with time. The results show that there was no nickel quenching by medium particles and all the nickel were accumulated by the two isolates

Response of the isolates to antibiotics

Table 5 and figure 6 indicates the antibiotype and resistant profile of both SAT-1 and SAT-2, study indicates that both the isolates were resistant for Ticarcilin and SAT-1 is also resistant for Ciprofloxacin and intermediate for Tetracyclin.

Assay of heavy metal content in fish flesh sample

Table 3 indicates that the concentration of both nickel and cadmium in the flesh flesh samples of both the species. Higher concentration of Cd was observed in *Glossogobius giuris* (2.19±0.5) µg g-1 dry wt, and in *Mystus cavasius* the amount was (1.53±0.3) µg g-1 dry wt, for nickel also the

higher concentration was found in *Glossogobius giuris* (13.61±1.74) µg g-1 dry wt and in *Mystus cavasius* the amount was (7.34±2.0) µg g-1 dry wt.

Table.1 Physico-chemical analysis of water samples, Mean ± Standard Error. (* microbiological counts are mean of triplicates)

Temp. (0C)	pH	Conductivity (µmhos/cm)	DO (mg/l)	BOD (mg/l)	Total Coliform *(MPN/100 ml)	Faecal Coliform *(MPN/100 ml)	Nickel content (mg/L)	Cadmium content (mg/L)
27±0.3	8.5±0.05	1523±2.7	6.1±0.28	39±0.7	$22.3x10^5$	$9.7x10^5$	3.82±0.12	1.83±0.01

Table.2 Morphological, physiological and biochemical characteristics of the isolates

Bacterial isolates	*SAT-1*	*SAT-2*
Biochemical tests		
Gram staining	+ve, coccobacilli, in clusters	-ve, bacilli, mostly single
Catalase	+	+
Oxidase	-	-
Amylase	+	+
Cellulase	-	-
Protease	-	-
Lipase	-	-
DNase	-	+
Endospore formation	-	-
Motility	+	+
Aerobic growth	++	++
Anaerobic growth	-	-
H2S production	-	-
Gelatin liquefaction	-	-
Carbohydrate utilization		
Glucose	+++	+++
Sucrose	++	++
Lactose	++	++

+indicates presence or positive reaction, - indicates absence or negative reaction

Table.3 Comparative Ni+2 MIC assay between the two isolates

	Ni+2 conc. (mM) in Luria broth												
Isolates	**Inoculum Blank**	**0.5**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
SAT-2	-	+	+	+	-	-	-	-	-	-	-	-	-
SAT-1	-	+	+	+	+	+	+	+	+	+	+	-	-

("+" denotes growth, "-"denotes no growth)

Table.4 Comparative Cd2+ MIC assay between the two isolates

	Cd+2 conc. (mM) in Luria broth												
Isolates	**Inoculum Blank**	**0.5**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
SAT-2	-	+	+	+	+	+	+	+	-	-	-	-	-
SAT-1	-	+	+	+	+	+	+	+	+	-	-	-	-

("+" denotes growth, "-"denotes no growth)

Table.5 Tests for antibiotic resistance of the two selected isolates

Antibiotic	***Amikacin***	***Imipenem***	***Ciprofloxacin***	***Polymyxin B***	***Colistin***	***Netilin***	***Tetracyclin***	***Ticarcilin***	***Gentamycin***
Isolates	*30 µg/ml*	*10 µg/ml*	*5 µg/ml*	*300µg/ml*	*10 µg/ml*	*30µg/ml*	*30µg/ml*	*75µg/ml*	*10µg/ml*
SAT1	**S**	**S**	**R**	**S**	**S**	**S**	**I**	**R**	**S**
SAT2	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**

Table.6 Heavy metal concentrations (range and mean) in muscle tissue of fishes. All results are expressed as Mean± Standard Error (SE) of Mean; n=10

Fish Species (no of samples: 10 for each species)	**Cadmium (FAO/WHO guidelines- 1µg g^{-1} dry wt)**	**Nickel (FAO/WHO guidelines- 10µg g^{-1} dry wt)**
Mystus cavasius	**(1.53±0.3) µg g^{-1} dry wt**	**(7.34±2.0) µg g^{-1} dry wt**
Glossogobius giuris	**(2.19±0.5) µg g^{-1} dry wt**	**(13.61±1.74) µg g^{-1} dry wt**

Fig.1 Map indicating the location of sample collection sites 1. (Baranagar)(22.64°N 88.37°E) 2. (Belur) (22°37′57″N 88°21′23″E) 3. (Kashipur)(22.623°N 88.375°E)

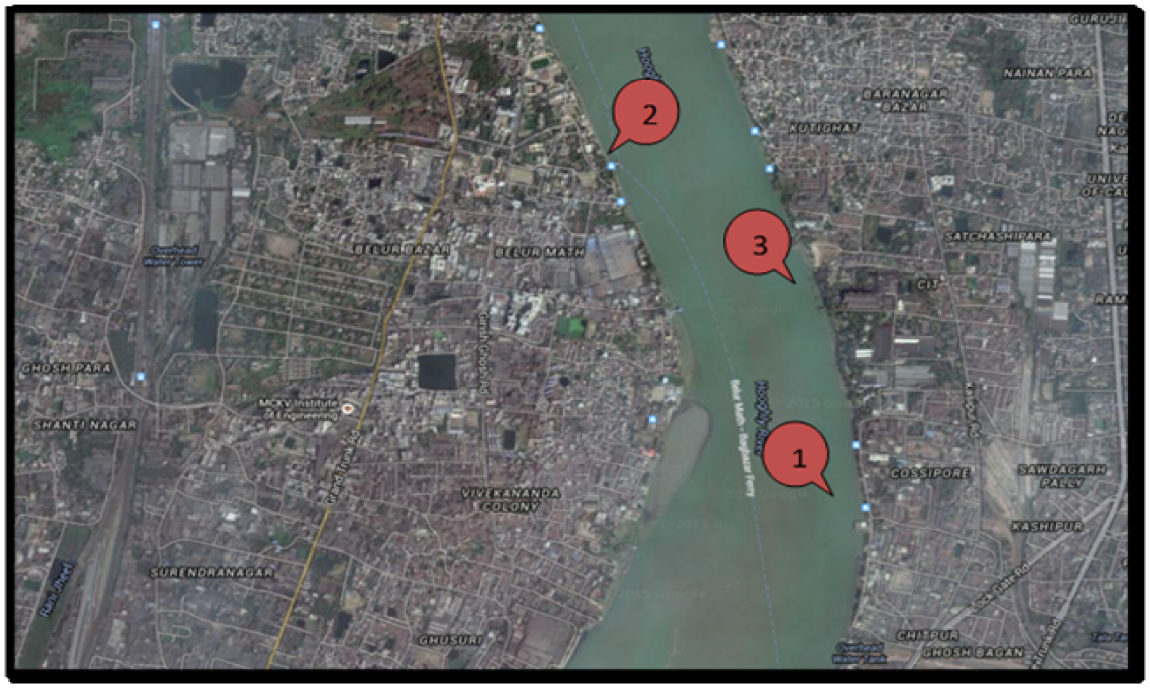


Fig.2 Amount of nickel content in the bacterial pellet, determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). The result is a mean of 5 assays and the graph shows mean profile with ±1 unit error

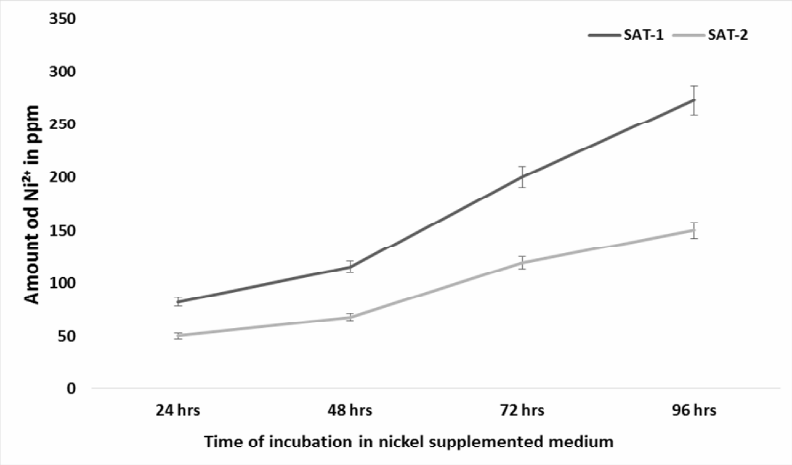


Fig.3 Amount of nickel content Nickel content in culture medium supernatant, determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). The result is a mean of 5 assays and the graph shows mean profile with ±1 unit error

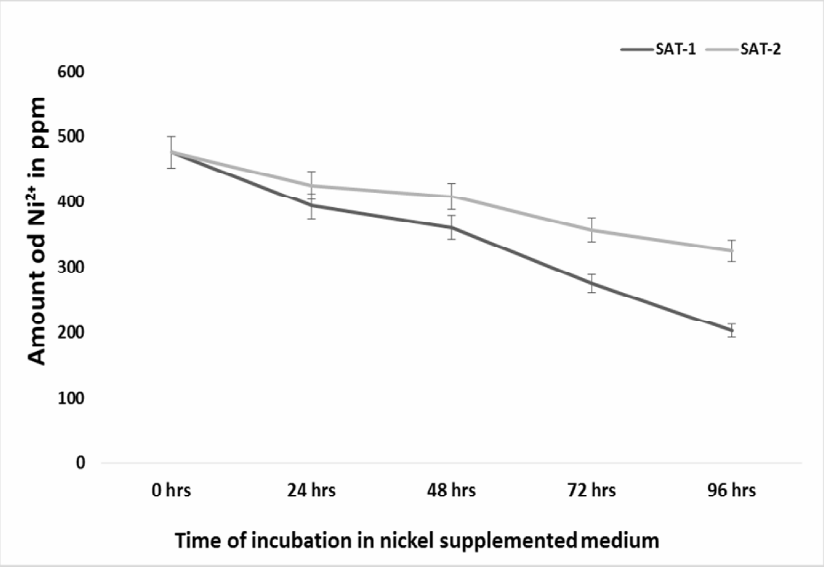


Fig.4 Amount of Cadmium content in the bacterial pellet, determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA).
The result is a mean of 5 assays and the graph shows mean profile with ±1 unit error

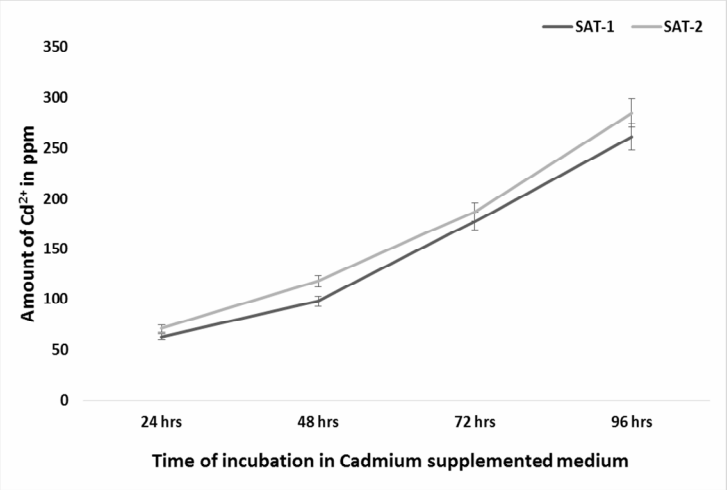


Fig.5 Amount of nickel content Cadmium content in culture medium supernatant, determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). The result is a mean of 5 assays and the graph shows mean profile with ±1 unit error

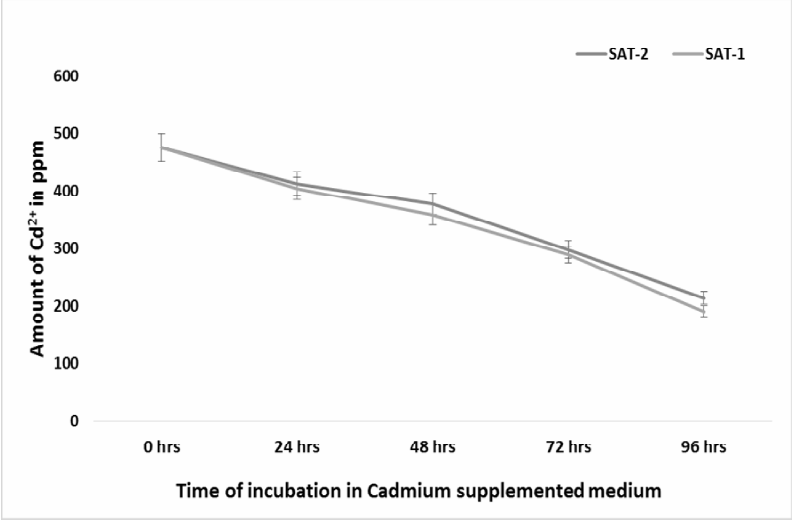
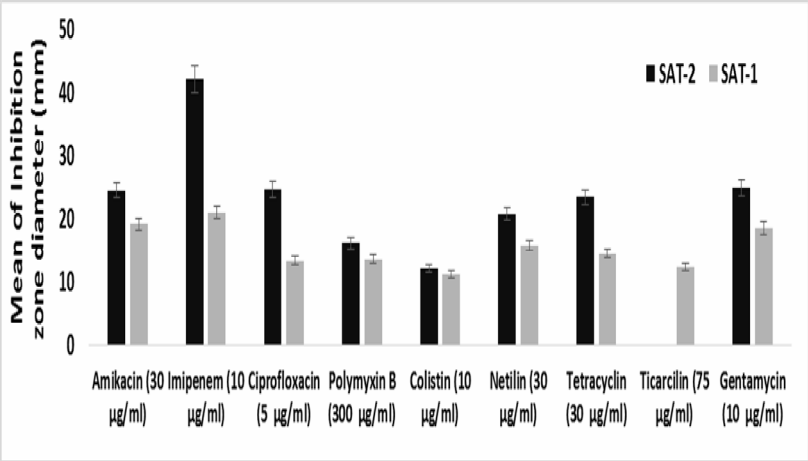


Fig.6 Comparative Antibiotypes of the isolates SAT-1 and SAT-2. The result is a mean of 5 assays and the graph shows mean profile with ±1 unit error



In conclusion, both *Mystus cavasius* and *Glossogobius giuris* are one of the fishery resources and mainly consumed by many people in the study area. Hence it is ascertain to know the suitability and safety for the local people regarding their edible fishes. This study was undertaken to view toxicological importance of these edible fishes to know its variations in heavy metals contamination. Study showed that there are both Nickel and Cadmium contamination in study area of Hooghly River's water samples and nickel and cadmium resistance in the bacteria from the fish samples were also found. Study also suggests that isolates SAT-1 and SAT-2 which have been isolated from *Mystus cavasius* and *Glossogobius giuris* respectively, both accumulate significant amount of nickel and cadmium, presence of such heavy metal accumulating bacterial strains in the gut may facilitate the absorption of heavy metals namely nickel and cadmium throughout the body and may also accumulate in the flesh of the fish which is the most edible part. Therefore, the consumption of this fishes contaminated with these bacteria pose a great risk for public health. When considering the heavy metals concentrations in fish species, the most important aspect is their toxicity to humans suitable for human consumption. The results of this study revealed that consuming Glossogobius giuris from the study area of the river Hooghly, India may be harmful to consumers because observed values of heavy metals were slightly higher than the permissible limits issued by FAO/WHO for human consumption. However, in case of Mystus cavasius, study indicates that the level of both nickel and cadmium levels is alarming to go beyond the permissible level. The heavy metals contamination not only affects the fish but also other aquatic life gets affected. Hence a more intensive study is needed in order to determine the bioaccumulation of heavy metals in fishes from the study area. Further study on accumulation of organochlorine pesticides, PCBs, PAHs, and dioxins in fish tissues should be undertaken due to usage of these chemicals in India.

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